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14. ABSTRACT

Prostate cancer is the most commonly diagnosed cancer and is the second leading cause of cancer death in the US. Most patients diagnosed with prostate cancer are treatable, but the patients usually die from hormone refractory (HRPC) and metastatic disease. We have previously shown that expression of Notch receptors and their ligands is upregulated in many cancers including prostate cancer. We hypothesize that inactivation of Jagged-1 signaling, which could be directly due to transcriptional inactivation of Jagged-1 or indirectly due to inactivation of Akt/NF-κB, will not only be a novel approach for the treatment of HRPC and metastases but will also sensitize prostate cancer cells to Taxotere-induced killing. We found that down-regulation of Notch-1 and Jagged-1 induced cell growth inhibition and cell apoptosis. We also found that down-regulation of Notch-1 and Jagged-1 decreased the expression and activities of VEGF, MMP-9, uPA. Moreover, down-regulation of Notch-1 and Jagged-1 inhibited the NF-κB DNA binding activity. Consistent with these results, we also found that the down-regulation of Notch-1 and Jagged-1 inhibited the cell migration and invasion in prostate cells. Collectively, our results suggest that down-regulation of Jagged-1 and Notch-1 could be useful strategy for treatment of prostate cancer. Based on above hypothesis, this proposal seems highly relevant to the mission of the Department of Defense.

15. SUBJECT TERMS
None provided.

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Introduction

Prostate cancer is the most commonly diagnosed cancer and is the second leading cause of cancer death in the US (1). Most patients diagnosed with prostate cancer are treatable, but the patients usually die from hormone refractory (HRPC) and metastatic disease. Thus there is a dire need for the development of novel strategies by which HRPC and metastatic disease could be treated with a better outcome. Among many signaling pathways, the Notch signaling pathway has recently emerged and appears to play a critical role in maintaining the balance between cell proliferation, differentiation and apoptosis. There are considerable evidences showing that expression of Notch receptors and their ligands is upregulated in many cancers including prostate cancer (2-10). Hence, perturbation in Notch signaling is believed to contribute to tumorigenesis. It has been reported that Jagged-1 is highly expressed in prostate cancer cells (11, 12). It was found to be increased in the conditioned media in prostate cancer cells (13). Jagged-1 is significantly over expressed in metastatic prostate cancer as compared with localized prostate cancer or benign prostatic tissues (14). Furthermore, high Jagged-1 expression in a subset of clinically localized tumors was significantly associated with recurrence, independent of other clinical parameters (14). These findings suggest that dysregulation of Jagged-1 protein levels plays a role in prostate cancer cell growth and progression to metastatic disease. Therefore, down-regulation of Jagged-1 signaling could be a novel approach for the treatment of HRPC and metastatic disease. Data from our laboratory showed that Jagged-1 signaling could be down regulated by Jagged-1 siRNA but more importantly by soy isoflavone genistein (a known chemopreventive agent) and that there is a cross talk between Akt/NF-κB and Jagged-1 signaling. We hypothesize that inactivation of Jagged-1 signaling by genistein, which could be directly due to transcriptional inactivation of Jagged-1 or indirectly due to inactivation of Akt/NF-κB, will not only be a novel approach for the treatment of HRPC and metastases but will also sensitize prostate cancer cells to Taxotere-induced killing. The purpose of our current investigation: is 1) to determine the effect of altered Jagged-1 expression on prostate cancer cells. We will determine the critical contribution of Jagged-1 to prostate cancer cell proliferation, migration and invasion by using the Jagged-1-siRNA and Jagged-1-cDNA transfection experiments for down-regulation and over-expression of Jagged-1, respectively. 2) to determine whether Jagged-1 over-expression contributes to prostate cancer progression via activation of Akt/NF-κB pathway. We will determine the molecular mechanisms by which Jagged-1 regulates NF-κB and their downstream signaling pathway (VEGF, MMP-2 and MMP-9), leading to apoptototic cell death and inhibition of invasion and angiogenesis. 3) to test whether the down regulation of Jagged-1 signaling by chemopreventive agents (genistein) could sensitize PC-3, DU145, LNCaP and C4-2B prostate cancer cells to Taxotere-induced cell growth inhibition and apoptosis, and we will also test whether the chemo-sensitizing effect of genistein is mechanistically associated with Jagged-1/NF-κB signaling and its downstream genes, especially MMP-9 and VEGF.

Body of report

The original statement of work in the proposal is listed below:

Task-1: To determine the effect of altered Jagged-1 expression on prostate cancer cells. We will determine the critical contribution of Jagged-1 to prostate cancer cell proliferation, migration and invasion by using the Jagged-1-siRNA and Jagged-1-cDNA transfection experiments for down-regulation and over-expression of Jagged-1, respectively (Month 1-8):

a) Conduct our experiments in Jagged-1 siRNA and Jagged-1 cDNA transfected prostate cancer cells to establish the mechanistic role of Jagged-1
b) The migration and invasion activity of Jagged-1 cDNA or Jagged-1 siRNA transfected cells will be tested

Task-2: To determine whether Jagged-1 over-expression contributes to prostate cancer progression via activation of Akt/NF-κB pathway. We will determine the molecular mechanisms by which Jagged-1 regulates NF-κB and their downstream signaling pathway (VEGF, MMP-2 and MMP-9), leading to apoptototic cell death and inhibition of invasion and angiogenesis (Month 9-16).

a) Test how Jagged-1 may NF-κB and its downstream genes such as MMP-2, MMP-9 and VEGF.
b) Test the consequence of MMP-9 and VEGF down regulation on prostate cancer cell angiogenesis.

Task-3: We will test whether the down regulation of Jagged-1 signaling by chemopreventive agents (genistein) could sensitize PC-3, DU145, LNCaP and C4-2B prostate cancer cells to Taxotere-induced cell growth inhibition and apoptosis, and we will also test whether the chemo-sensitizing effect of genistein is mechanistically associated with Jagged-1/NF-κB signaling and its downstream genes, especially MMP-9 and VEGF (Month 17-24).

a) Conduct our experiments in Jagged-1 siRNA and Jagged-1 cDNA transfected prostate cancer cells treated with or without genistein to establish the mechanistic role of Jagged-1 during genistein-induced cell growth inhibition and induction of apoptosis.
b) Prostate cancer cells will be exposed to isoflavone for 24 hours followed by treatment with Taxotere. We will measure cell growth inhibition by MTT assay and apoptosis by histone/DNA ELISA.
c) Proteins extracted from the cells will be subjected to Western blot analysis for measuring the levels of Jagged-1 and NF-κB targeted genes (such as Jagged-1, Notch-1, MMP-9, VEGF, COX-2).
d) Measure the DNA binding activity of NF-κB, and all the signals will be quantitated following our standard approach.

We are providing the evidences to support the research proposal. We have completed majority parts of task 1 and task 2 and plan to complete the rest of task this year. We are making progress on data analysis and manuscript writing now.

We are now reporting the research accomplishments associated with task 1 and 2 outlined in the State of Work.

Material and Methods:

Cell culture and experimental reagents. Human PC cell lines, including PC-3, DU145, LNCaP, C4-2B and MDA PCa2B cells, were grown in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS), 100 μg/ml streptomycin, 100 units/ml penicillin, and 2.5 mM glutamine in a humidified incubator with 5% CO_2 and 95% air at 37°C. Primary antibodies for Notch-1, Notch-2, Notch-3, Notch-4, Jagged-1, Jagged-2, Dll-1, Dll-4, uPA, uPAR, VEGF and MMP-9 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against mTOR, phospho-mTOR (Ser^{2448}), p70S6K, phospho-p70S6K (Thr^{389}), 4E-BP1, phospho-4E-BP1 (Thr^{37}/Thr^{46}), Akt, and phospho-Akt (Ser^{473}) were purchased from Cell Signaling Technology. The monoclonal antibody to β-actin and PI3K inhibitor, LY294002, were purchased from Sigma-Aldrich. All secondary antibodies were obtained from Pierce (Rockford, IL). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA). Chemiluminescence detection of proteins was done with the use of a kit from Amersham Biosciences (Amersham Pharmacia Biotech, Piscataway, NJ). Protease inhibitor cocktail, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and all other chemicals were obtained from Sigma (St. Louis, MO).

Plasmids and transfections. The Notch-1 cDNA plasmid encoding the Notch-1 intracellular domain and Jagged-1 cDNA plasmid were the kind gifts of L. Miele {Department of Biopharmaceutical Sciences and Cancer Center, University of Illinois at Chicago, Chicago, IL; ref). PC cells were transfected with Notch-1 siRNA, Jagged-1 siRNA and siRNA control, respectively, using Lipofectamine 2000.

Cell growth inhibition studies by WST-1 assay. The transfected prostate cancer cells (5×10^3) were seeded in a 96-well culture plate. After incubation for 24, 48, and 72 h, the cells were incubated with cell proliferation reagent WST-1 (Roche Applied Science) in medium for 4 hours at 37°C and 5% CO_2. The spectrophotometric absorbance was determined by using Ultra Multifunctional Microplate Reader (Tecan) at 450/595 nm. In addition to the above assay, we have also done clonogenic assay for assessing the effects of treatment as shown below.

Clonogenic assay. To test the survival of transfected cells, siRNA transfected PC-3 cells were trypsinized, and the viable cells were counted (trypan blue exclusion) and plated in 100 mm Petri dishes in a range of 100 to 1,000 cells to determine the plating efficiency as well as for assessing the effects of transfection on clonogenic survival. The cells were then incubated for 10 to 12 days at 37°C in a 5% CO_2/5% O_2/90% N_2 incubator. The colonies were stained with 2% crystal violet and counted. The surviving fraction was normalized to untreated control cells with respect to clonogenic efficiency.

Histone/DNA ELISA for detection of apoptosis. The Cell Death Detection ELISA Kit was used for assessing apoptosis according to the manufacturer's protocol. Briefly, transfected cells were lysed and the cell lysates were overlaid and incubated in microtiter plate modules coated with anti-histone antibody for detection of apoptosis as described earlier.

Real-time reverse transcription-PCR analysis for gene expression studies. The total RNA from transfected cells was isolated by Trizol (Invitrogen, Carlsbad, CA) and purified by RNeasy Mini Kit and RNase-free DNase Set (QIAGEN, Valencia, CA) according to the manufacturer's

protocols. One microgram of total RNA from each sample was subjected to first strand cDNA synthesis using TaqMan reverse transcription reagents kit (Applied Biosystems, Foster City, CA) in a total volume of 50 microliters, including 6.25 U MultiScribe reverse transcriptase and 25 pmol random hexamers. RT reaction was performed at 25ºC for 10 min, followed by 48ºC for 30 min and 95ºC for 5 min. The primers used in the PCR reaction for Notch-1, Jagged-1, MMP-9, VEGF, uPA and β-actin were described before. Real-time PCR amplications were performed as described earlier.

Western blot analysis. Cells were lysed in lysis buffer [50 mmol/L Tris (pH 7.5), 100 mmol/L NaCl, 1 mmol/L EDTA, 0.5% NP40, 0.5% Triton X-100, 2.5 mmol/L sodium orthovanadate, 10 μL/mL protease inhibitor cocktail and 1 mmol/L PMSF] by incubating for 20 minutes at 4°C. The protein concentration was determined using the Bio-Rad assay system (Bio-Rad, Hercules, CA). Total proteins were fractionated using SDS-PAGE and transferred onto a nitrocellulose membrane for Western blotting as described before.

Urokinase plasminogen activator (uPA) activity assay. The culture medium of the transfected cells grown in 6-well plates was collected. After collection, the medium was spun at 800 x *g* for 3 min at 4°C to remove cell debris. The supernatant was either frozen at -20°C for uPA assay later or assayed immediately using commercially available ELISA kits (American Diagnostica, Inc., Stamford, CT).

MMP-9 activity assay. The transfected cells were seeded in 6 well plates and incubated at 37°C. After 24 hours, the complete medium was removed and the cells were washed with serum-free medium. The cells were then incubated in serum-free medium for 48 hours. MMP-9 activity in the medium and cell lysate was detected by using Fluorokine E Human MMP-9 Activity Assay Kit (R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's protocol.

VEGF assay. The transfected cells were seeded in 6 well plates (1.0×10^5 cells per well) and incubated at 37°C. After 24 hours, the cell culture supernatant was harvested and cell count was performed after trypsinization. After collection, the medium was spun at 800 x *g* for 3 min at 4°C to remove cell debris. The supernatant was either frozen at -20°C for later VEGF assay or assayed immediately using commercially available ELISA kits (R&D Systems, Inc., Minneapolis, MN).

Cell migration and invasion assay. Cell migration was assessed using 24-well inserts (BD Biosciences, Bedford, MA) with 8μm pores according to the manufacturer's protocol. The invasive activity of the GR siRNA transfected cells was tested using the BD BioCoat Tumor Invasion Assay System (BD Biosciences, Bedford, MA) as described before. Briefly, transfected PC-3 cells (5×10^4) with serum free medium were seeded into the upper chamber of the system. Bottom wells in the system were filled with complete medium. After 24 hours of incubation, the cells in the upper chamber were removed, and the cells, which invaded through matrigel matrix membrane, were stained with 4 μg/ml Calcein AM in Hanks buffered saline at 37ºC for one hour. Then, fluorescence of the invaded cells was read in ULTRA Multifunctional Microplate Reader (TECAN) at excitation/emission wavelengths of 530/590 nm. These fluorescently labeled invasive cells were also photographed under a fluorescent microscope.

Densitometric and statistical analysis. The statistical significance of differential findings between experimental groups and control was statistically evaluated using GraphPad StatMate software (GraphPad Software, Inc., San Diego, CA). *P* values lower than 0.05 were considered statistically significant.

Results:

1. Notch signaling pathway in prostate cancer cells. The baseline expression and activation of the Notch signaling mRNA and proteins were determined in a panel of human prostate cancer cell lines that included PC-3, DU145, LNCaP, C4-2B, and MDA PCa2B. The results showed that the Notch signaling pathway was frequently but differentially dysregulated in the different human prostate cancer cell lines (Fig-1). This information will be important in interpreting data on the experiments below.

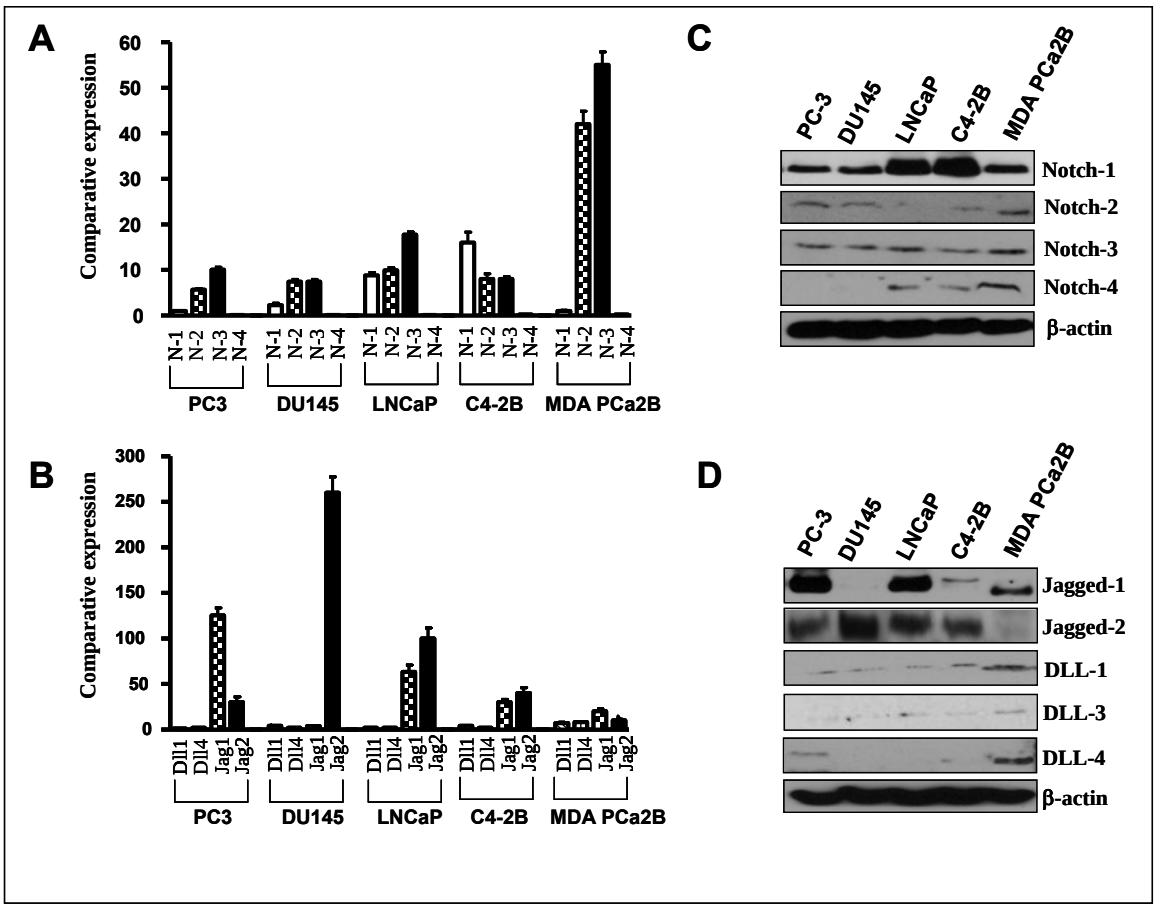


Fig-1: The baseline expression of the Notch signaling mRNA and proteins were determined in a panel of human prostate cancer cell lines. Notch signaling pathway was frequently but differentially dysregulated in the different human prostate cancer cell lines.

2. Down-regulation of Jagged-1 decreases the expression of Notch receptors. To investigate which receptors were affected by Jagged-1 siRNA, we detected the expression of Notch receptors after Jagged-1 siRNA transfection using Western blot analysis. The efficacy of siRNA for knockdown of Jagged-1, Notch mRNA and protein was confirmed by real-time RT-PCR and Western blotting. We observed that both Jagged-1 and Notch-1 mRNA and protein levels were barely detectable in Jagged-1 siRNA or Notch-1 siRNA transfected cells, compared to siRNA control transfected cells (Fig. 2). However, over-expression of Jagged-1 was found in the Jagged-1 cDNA transfected DU145 and C4-2B cells. To investigate which receptors were affected by Jagged-1 siRNA, we detected the expression of Notch receptors in PC-3 and LNCaP cells after Jagged-1 siRNA transfection using Western blot analysis. We found that Notch receptors were decreased in Jagged-1 transfected cells.

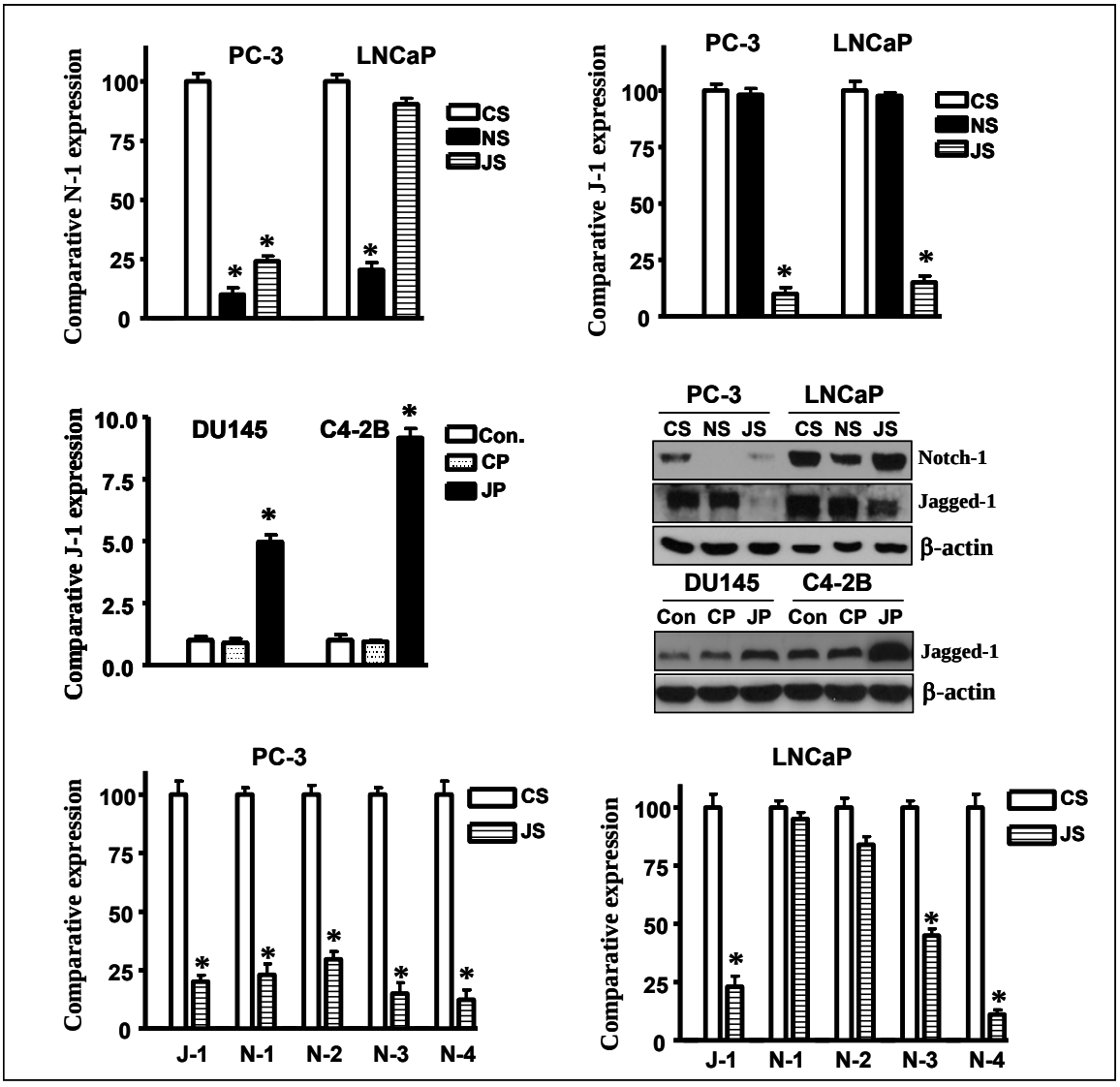


Fig-2: The efficacy of siRNA for knockdown of Jagged-1, Notch mRNA and protein was confirmed by real-time RT-PCR and Western blotting. Both Jagged-1 and Notch-1 mRNA and protein levels were barely detectable in Jagged-1 siRNA or Notch-1 siRNA transfected cells, compared to siRNA control transfected cells. However, over-expression of Jagged-1 was found in the Jagged-1 cDNA transfected DU145 and C4-2B cells.

3. Down-regulation of Jagged-1 or Notch-1 induces cell growth inhibition. To determine whether Notch could be an effective therapeutic target for prostate cancer, the effect of Notch1, Jagged-1 siRNA on cell growth of the prostate cancer cells was examined. The cell viability was determined by MTT and the effect of Jagged-1 siRNA on the growth of cancer cells is shown on Figure. 3. We found that down-regulation of Jagged-1 expression caused cell growth inhibition in prostate cancer cell lines. However, over-expression of Jagged-1 promotes cell growth in DU145 and C4-2B. To determine the effect of Notch and Jagged-1 on cell growth, transfected cells were also assessed for cell viability by clonogenic assay. Down-regulation of Notch-1 and Jagged-1 resulted in a significant inhibition of colony formation of PC-3 cells when compared with control (Fig. 3).

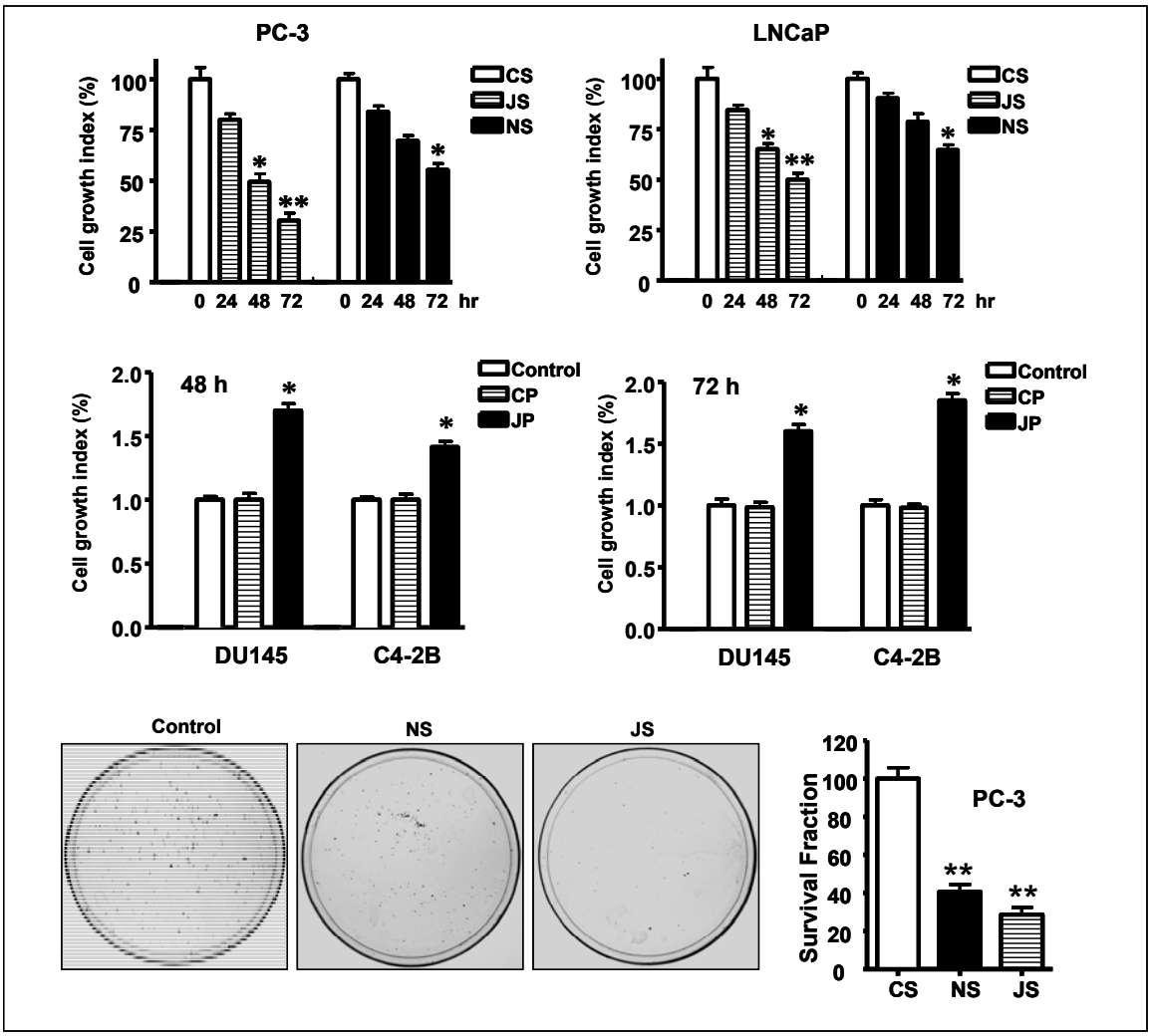


Fig-3: Down-regulation of Notch-1 and Jagged-1 expression caused cell growth inhibition in prostate cancer cell lines. However, over-expression of Jagged-1 promotes cell growth in DU145 and C4-2B. Down-regulation of Notch-1 and Jagged-1 resulted in a significant inhibition of colony formation of PC-3 cells when compared with control.

4. Down-regulation of Jagged-1 induced apoptosis in prostate cancer cell lines. To quantitatively measure apoptotic cell death after transfection, we conducted a histone/DNA enzyme-linked immunosorbent apoptosis assay. We found that down-regulation of Jagged-1 induced apoptosis. However, over-expression of Jagged-1 by cDNA transfection inhibited apoptosis.

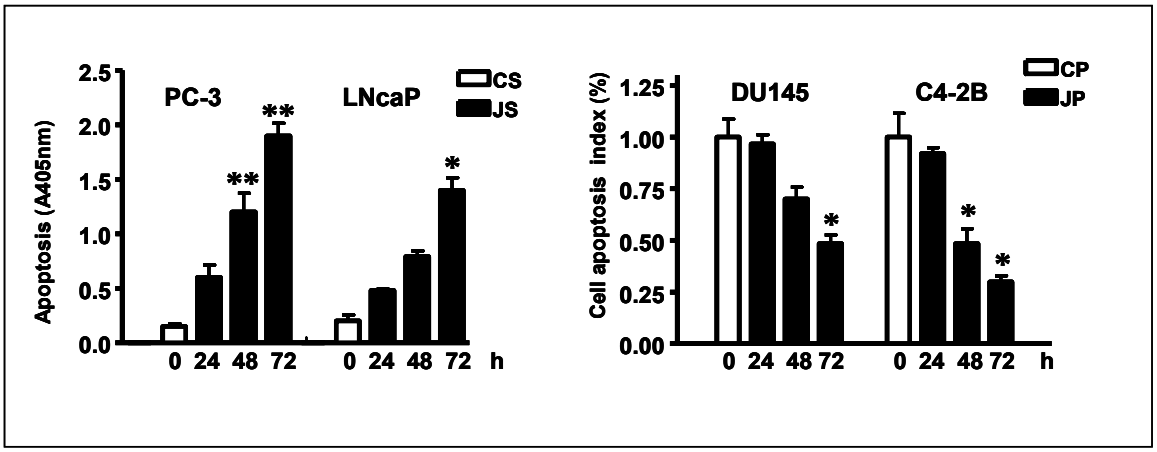
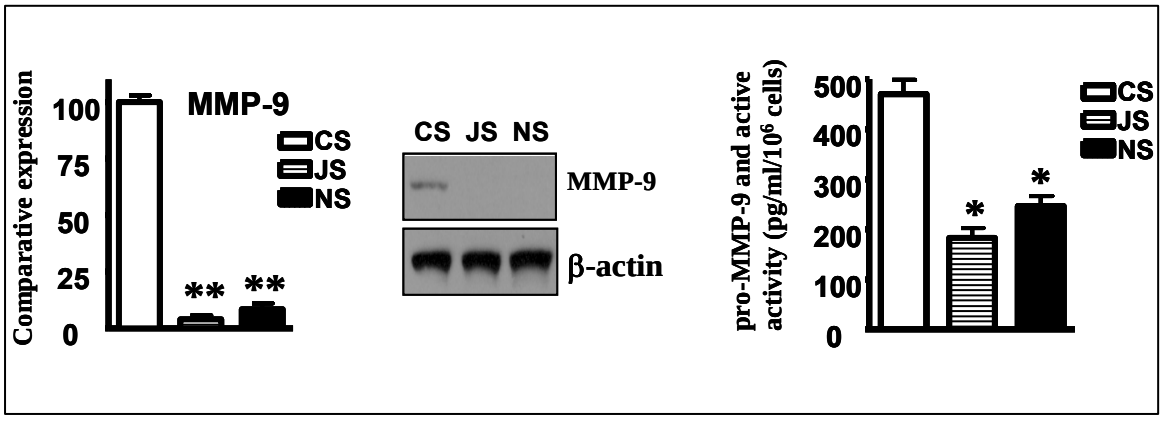


Fig-4: Down-regulation of Jagged-1 induced apoptosis. However, over-expression of Jagged-1 inhibited apoptosis.

5. Down-regulation of Notch-1 and Jagged-1 decreased MMP-9 gene transcription and their activities. We therefore investigated whether MMP-9 were down-regulated by Jagged-1 and Notch-1 siRNA in prostate PC-3 cancer cell line. To explore whether Jagged-1 and Notch-1 siRNA transfection could decrease the expression of MMP-9, real-time RT-PCR and Western blotting were conducted. We found that both MMP-9 mRNA and protein levels were dramatically decreased in the Notch-1 and Jagged-1 siRNA transfected cells (Fig. 5). Next, we examined whether the down-regulation of Jagged-1 and Notch-1 could lead to a decrease in MMP-9 activity in prostate cancer cells. There was a marked decrease in the activity of MMP-9 in siRNA transfected cells.



Fjg-5: Down-regulation of Notch-1 and Jagged-1 decreased MMP-9 gene transcription and their activities.

6. Notch-1 siRNA and Jagged-1 siRNA reduced uPA gene transcription and translation. To further investigate whether Notch-1 and Jagged-1 siRNA have any effect on reducing the level of uPA, real-time RT-PCR and Western blotting were done to detect the expression of uPA. We found that both uPA mRNA and protein levels were dramatically reduced in Notch-1 and Jagged-1 siRNA transfected cells (Fig. 6).

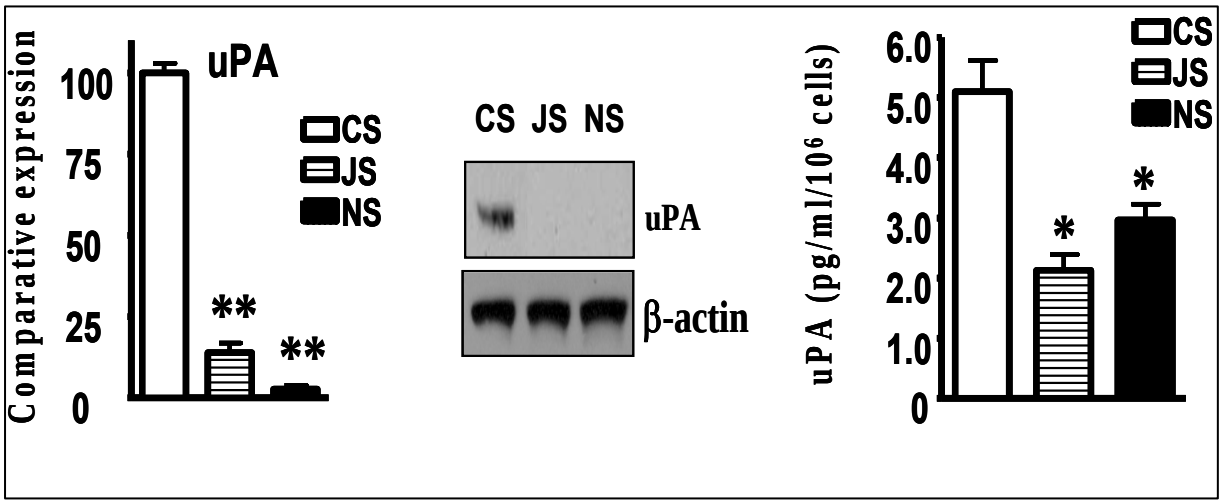


Fig-6: Down-regulation of Notch-1 and Jagged-1 decreased uPA gene transcription, translation and their activities.

7. Notch-1 and Jagged-1 siRNA decreased VEGF activity. To further explore whether Notch-1 siRNA reduced VEGF activity, we examined the levels of VEGF activity secreted in the culture medium. We found that Notch-1 and Jagged-1 siRNA could lead to a decrease in the levels of VEGF secreted in the culture medium (Fig. 7).

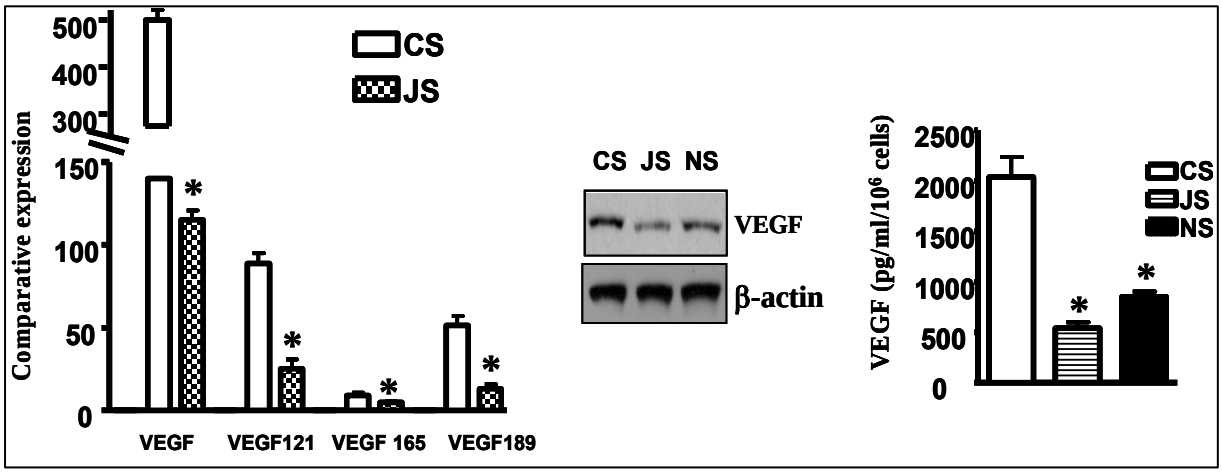


Fig-7: Down-regulation of Notch-1 and Jagged-1 decreased VEGF activity

8. Down-regulation of Notch-1 and Jagged-1 decreased NF-κB DNA binding activity. Notch signal pathway has been reported to cross-talk with NF-κB signaling pathway. Therefore, we measured the NF-κB DNA-binding activity by EMSA in Notch-1 and Jagged-1 siRNA transfected cells. We found that down-regulation of Notch-1 and Jagged-1 by siRNA transfection decreased NF-κB DNA-binding activity (Fig. 8).

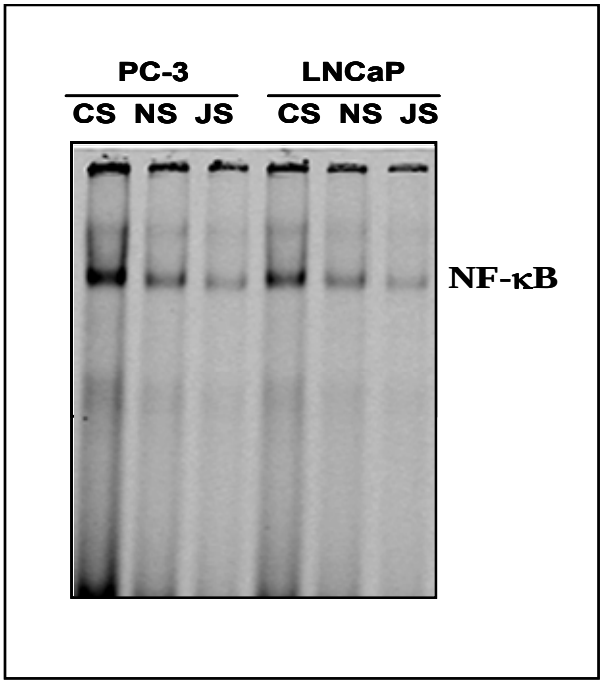


Fig-8: Down-regulation of Notch-1 and Jagged-1 decreased NF-κB DNA binding activity. The transfected cells were washed with cold phosphate-buffered saline and suspended in 0.15 ml of lysis buffer [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF, 2 µg/ml leupeptin, 2 µg/ml aprotinin, and 0.5 mg/ml benzamidine]. The nuclear protein was prepared and subjected to DNA binding activity of NF-κB by EMSA.

9. Down-regulation of Notch-1 and Jagged-1 decreased PC-3 prostate cancer cell migration and invasion. MMP-9, VEGF and uPA are thought to be critically involved in the processes of tumor cell migration, invasion and metastasis. Because Notch-1 and Jagged-1 siRNA inhibited the expression and activity of MMP-9, VEGF and uPA, we tested the effects of Notch-1 and

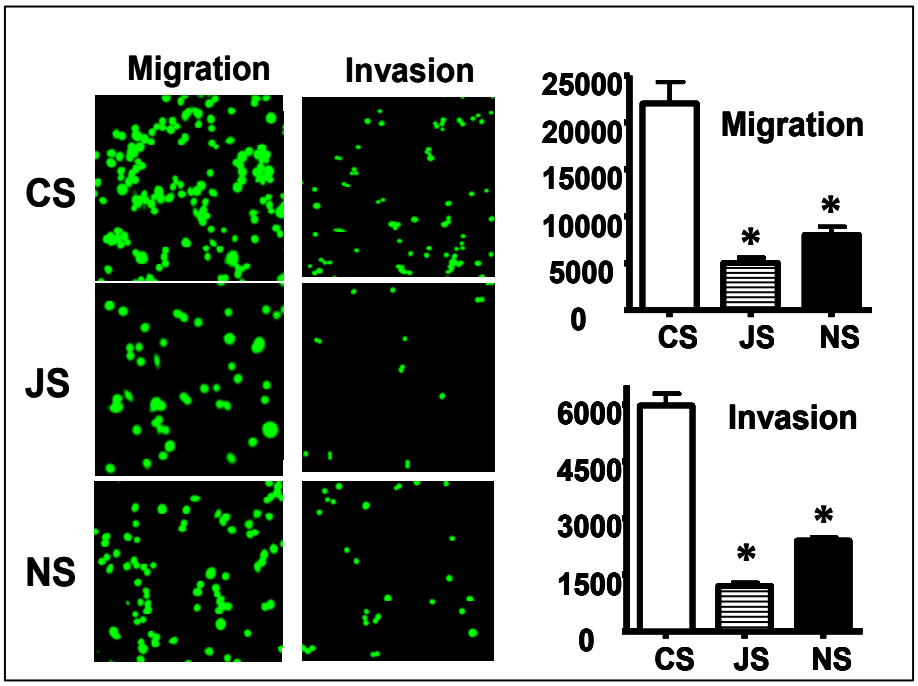


Fig-9: Down-regulation of Notch-1 and Jagged-1 decreased PC-3 prostate cancer cell migration and invasion.

Jagged-1 down-regulation on cancer cell migration and invasion. We found that down-regulation of Notch-1 and Jagged-1 decreased prostate cancer cell migration. Moreover, as illustrated in Figure 9, Notch-1 and Jagged-1 siRNA transfected cells showed a low level of penetration through the matrigel-coated membrane compared with the control cells. The value of fluorescence from the invaded PC-3 prostate cancer cells was decreased about 3-4 fold compared with that of control cells (Fig. 9).

Key Research Accomplishments

We have done several key experiments, such as transfection, ELISA. We do not have any technical difficulties because we have conducted these types of experiments.

Reportable Outcomes

We are making progress on data analysis and manuscript writing now.

Conclusions

- **We have found that Notch signaling pathway plays important roles in prostate cancer cells.**
- **Down-regulation of Jagged-1 or Notch-1 induces cell growth inhibition.**
- **Down-regulation of Jagged-1 induced apoptosis in prostate cancer cell lines.**
- **Down-regulation of Notch-1 and Jagged-1 decreased MMP-9 gene transcription and their activities.**
- **Notch-1 siRNA and Jagged-1 siRNA reduced uPA gene transcription and translation.**
- **Notch-1 and Jagged-1 siRNA decreased VEGF activity.**
- **Down-regulation of Notch-1 and Jagged-1 decreased NF-κB DNA binding activity.**
- **Down-regulation of Notch-1 and Jagged-1 decreased PC-3 prostate cancer cell migration and invasion.**

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